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# Expression of Vascular Permeability Factor/Vascular Endothelial Growth Factor by Melanoma Cells Increases Tumor Growth, Angiogenesis, and Experimental Metastasis<sup>1</sup>

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## ABSTRACT

Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) is an angiogenic cytokine expressed by many human and animal tumors. Hypoxia often up-regulates VPF/VEGF expression further. To better define the role of VPF/VEGF in tumor biology, we screened tumorigenic lines for those expressing minimal constitutive and hypoxia-inducible VPF/VEGF. Human melanoma SK-MEL-2 cells best fit these criteria and formed small, poorly vascularized tumors in immunodeficient mice. We transfected SK-MEL-2 cells stably with sense or antisense mouse VPF/VEGF cDNA or with vector alone. Cells transfected with sense VPF/VEGF (V+) expressed and secreted large amounts of mouse VPF/VEGF and formed well-vascularized tumors with hyperpermeable blood vessels and minimal necrosis in nude/SCID mice. Antisense-transfected VPF/VEGF (V-) cells expressed reduced constitutive VPF/VEGF and no detectable mouse VPF/VEGF, and formed small, minimally vascularized tumors exhibiting extensive necrosis. Vector-alone transfectants (N1 cells) behaved like parental cells. V+ cells formed numerous lung tumor colonies in SCID mice, ~50-fold more than N1 cells, whereas V- cells formed few or none. These experiments demonstrate that VPF/VEGF promotes melanoma growth by stimulating angiogenesis and that constitutive VPF/VEGF expression dramatically promotes tumor colonization in the lung.

## INTRODUCTION

VPF/VEGF<sup>3</sup> is a glycosylated, multifunctional cytokine that is abundantly expressed and secreted by most human and animal tumors examined thus far (1-10). It exerts a number of important biological actions on endothelial cells: (a) it acts with a potency some 50,000 times that of histamine to increase the permeability of microvessels to circulating macromolecules (1, 2, 8), and this activity likely accounts for the well-documented hyperpermeability of tumor blood vessels (8, 11-13); and (b) VPF/VEGF is a selective endothelial cell mitogen (14-21). In addition, VPF/VEGF alters endothelial cell gene expression, inducing increased production of tissue factor and several proteases, including interstitial collagenase and both the urokinase-like and tissue plasminogen activators (22, 23). VPF/VEGF potently promotes angiogenesis in both the chick chorioallantoic membrane and rabbit corneal assays (14, 24). Collectively, these several activities suggest important roles for VPF/VEGF in the induction of tumor angiogenesis.

Human VPF/VEGF is a *M<sub>r</sub>* 35,000-43,000 dimeric polypeptide that may be expressed in several isoforms (121, 165, 189, and 205 amino acids) resulting from alternative splicing of a single primary

transcript (25). Closely homologous transcripts are expressed in the mouse but contain one less amino acid (26). VPF/VEGF mRNAs contain a signal sequence that directs protein secretion, and the 165-amino acid transcript (164 amino acids in the mouse) is generally the most abundant secreted form. In contrast, the larger isoforms of VPF/VEGF remain predominantly cell associated (27, 28). VPF/VEGF is abundantly expressed by most tumorigenic cells, both *in vivo* and *in vitro*, but expression can be further increased by hypoxia and by several cytokines (29-37).

VPF/VEGF is thought to exert its several effects by interacting with two high affinity tyrosine kinase receptors (flt-1 and KDR/flk-1) that are selectively expressed in vascular endothelium (38-41). Like VPF/VEGF, both VPF/VEGF receptors are overexpressed in many human and animal tumors (4-6, 9, 42). Recent studies have also shown that VPF/VEGF and its receptors are overexpressed in a number of nonneoplastic pathologies and in certain physiological processes that, like tumors, are characterized by microvascular hyperpermeability, angiogenesis, and stroma formation; examples include normal wound healing (43, 44), rheumatoid arthritis (45, 46), psoriasis (35), corpus luteum formation (47, 48), endometrial cycling (49), and primary vasculogenesis as it occurs during embryonic development (44, 50, 51). Therefore, VPF/VEGF and VPF/VEGF receptor expression are closely linked to new blood vessel formation as it develops in a wide variety of disease states and in normal physiology.

The overexpression of VPF/VEGF by a wide spectrum of tumor cells has suggested an important role for this cytokine in tumorigenesis. In support of this view, Kim *et al.* (52) demonstrated that systemic treatment of tumor-bearing mice with a neutralizing antibody to VPF/VEGF significantly reduced tumor growth and correlated this effect with reduced tumor vascularity. Moreover, overexpression of a dominant-negative VPF/VEGF receptor (flk-1) was found to substantially reduce tumor growth and angiogenesis (53). On the other hand, VPF/VEGF transfection did not lead to cell transformation (54).

The goal of the present experiments was to investigate further the role of VPF/VEGF expression in primary tumor growth and angiogenesis and to begin to explore the possible role of this cytokine in tumor metastasis. To these ends we screened a number of cell lines, seeking tumorigenic cells that expressed minimal amounts of VPF/VEGF under both normoxic (constitutive expression) and hypoxic (induced expression) conditions. We identified a human melanoma cell line that best met these criteria and transfected it with mouse VPF/VEGF sense or antisense cDNA. We then related the growth and vascularity of these transfected tumor cells in immunodeficient nude or SCID mice to their expression of VPF/VEGF.

## MATERIALS AND METHODS

**Cell Culture, Transfection, and Selection.** The human melanoma cell line M21 was obtained from Dr. Romaine Saxton (UCLA, Los Angeles, CA), and

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<sup>3</sup> The abbreviations used are: VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; CMV, cytomegalovirus; FITC-D, FITC-labeled dextran.

SK-MEL-2 was obtained from American Type Culture Collection (Rockville, MD). Cell lines were cultured in DMEM containing 10% FCS, 2 mM/liter L-glutamine, 10 units/ml penicillin, and 10  $\mu$ g/ml streptomycin. Hypoxia experiments were performed with the BBL Gas-Pak system (Fisher Scientific, Pittsburgh, PA) using a palladium catalyst for anoxia with CO<sub>2</sub>. Cells were cultured in catalyst-activated chambers for periods of 4 or 24 h, resulting in a reported fall in pO<sub>2</sub> to 2% at 24 h (55).

The full-length mouse VPF/VEGF 164-amino acid cDNA (980 bp; Ref. 26) was cloned into an expression vector (pCMV-NEO) where transcription is driven by a CMV enhancer-promoter and contains a multiple cloning site, SV40 small t-intron and polyadenylation sequence, and a neomycin/Geneticin-sulfate (G418) selection cassette composed of the *Tn5* gene driven by the SV40 promoter. VPF/VEGF sense- and antisense-oriented constructs were analyzed by restriction mapping and by direct sequencing using the Sanger dideoxy method. DNA transfections were performed by calcium-phosphate precipitation as described previously (26, 56) using vector alone and sense and antisense VPF/VEGF constructs. Transfections were performed with 10  $\mu$ g circular-supercoiled DNA/100-mm culture dish. Forty-eight h after transfection, cells were split 1:3 into complete culture media containing 800  $\mu$ g/ml G418 to select transfectants. Twenty or more transected colonies were pooled, and cells were maintained in complete culture media with 500  $\mu$ g/ml G418 to assure DNA integration and expression.

**RNA Isolation and Northern Blot Analysis.** Total cellular RNA was isolated from cultured cell lines as described previously (26, 57). Northern blot analyses were performed using BioTrans nylon supported membranes (ICN, Irvine, CA) as described by the manufacturer. Mouse VPF/VEGF<sub>164</sub> cDNA probe was the 980-bp fragment isolated and described previously (26). The cDNA for a human ribosome-associated protein (36B4) probe (58) was used to control for RNA loading, blotting, and hybridization. Hybridization probes were isolated cDNA fragments radiolabeled with a random-primed synthesis kit (Multi-Prime; Amersham, Arlington Heights, IL). Blots were washed at high stringency (0.1 $\times$  SSC-1% SDS at 65°C) and exposed on Kodak X-OMAT film.

**Assays for VPF/VEGF Protein in Cell Culture Supernatants.** Immunoreactive VPF/VEGF was analyzed by our previously described fluorescence-linked immunoassay (59) modified to specifically detect mouse VPF/VEGF with an antipeptide antibody made to rat VPF/VEGF NH<sub>2</sub>-terminal amino acids 1–25, which detects both rat and mouse protein (60, 61). Human VPF/VEGF protein was detected with an anti-human VPF/VEGF NH<sub>2</sub>-terminal antibody described previously (7, 62, 63).

To evaluate the bioactivity of secreted VPF/VEGF, we made use of the Miles assay adapted to mouse skin (64). Serum-free conditioned media (24 h in DMEM, 6 mM/liter L-glutamine, 10 units/ml penicillin, and 10  $\mu$ g/ml streptomycin) was obtained from stable transected cells and normalized to each other by assaying total cellular protein extracted from the plate; protein levels from all dishes were less than 5% variation from each other. C57/Bl6 mice were injected i.v. with Evans blue dye (0.5% in PBS, 0.1 ml). After 10 min, test and control samples (0.1 ml) were injected intradermally into the flank. Twenty min later, mice were sacrificed, flank skin was removed, the underside was inspected, and test sites were photographed with a photomicroscope. Each sample was tested in at least three different mice.

**Microvascular Permeability in Tumor Xenografts.** Two methods were used to assess microvascular permeability in melanoma xenografts growing in the s.c. space of SCID mice. For macroscopic evaluation of macromolecular leakage, flank hair was removed with a clipper, and 16 h later, mice were injected i.v. with 0.1 ml of 0.5% Coomassie blue dye in PBS via the tail vein. Coomassie blue is a plasma albumin-binding dye that differs from Evan's blue in that it is cleared from the animal over the course of 2–4 h (65); as a result, repeated measurements of permeability were possible in a single animal by successive dye injections. Tumor sites were evaluated for dye extravasation and photographed 30 min after each i.v. injection of Coomassie blue dye.

Extravasation of circulating macromolecules at sites of tumor growth was determined microscopically using FITC-D as a tracer (11, 66). To remove any low molecular weight contaminants, FITC-D (*M<sub>r</sub>* 145,000; Sigma Chemical Co.) dissolved in PBS (30 mg/ml) was passed over a G-50 Sephadex (Pharmacia) column equilibrated with PBS, and the void volume was collected.

Tumor xenograft-bearing SCID mice were injected i.v. with 0.1 ml of FITC-D and sacrificed 30 min later. Animals were then exsanguinated and tumors with adjacent skin, and control tissues were suspended in alcohol:paraformaldehyde:PBS (70:4:26), a fixative that precipitates FITC-D, thereby preventing its further diffusion. Tissues were further dehydrated, embedded in paraffin, and sectioned for microscopy as described previously.

**Growth of Stably Transfected Melanoma Xenografts in Immunodeficient Mice.** Pooled and stably transfected SK-MEL-2 human melanoma cells ( $5 \times 10^5$  cells/site) were injected into the s.c. space of immunodeficient female nude mice (NCR-nu; Taconic Farms, Germantown, NY). Each animal received one such injection in each flank. In parallel experiments, similarly transfected SK-MEL-2 cells were injected into the deep dermis of female SCID mice (Taconic Farms, Germantown, NY), six sites/animal. For these experiments,  $2 \times 10^5$  cells were injected with a 30 gauge needle. s.c., and dermal tumors were harvested at 10–21 days and weighed individually prior to fixation.

In other experiments,  $2 \times 10^5$  tumor cells were injected i.v. in either SCID or NCR-nu mice. Animals were euthanized 21 days later, and lungs were harvested for evaluation of tumor colonies. Lungs were inflated with 4% paraformaldehyde-PBS, and external tumor nodules were counted with a dissecting microscope. Statistical analysis of tumor weights and numbers of lung colonies was performed with the Tukey-Kramer multiple comparison test.

**Histology, *In Situ* Hybridization, and Immunohistochemistry.** For routine histology, tumors and control tissues were fixed in 4% paraformaldehyde-PBS and processed and embedded in paraffin for hematoxylin and eosin staining. For *in situ* hybridization, tissues were fixed in cold 4% paraformaldehyde-PBS tissues for  $\leq 4$  h and transferred to sucrose (30% sucrose in 0.1 M PBS) for 16 h at 4°C. Tissues were then embedded in OCT compound, and 4- $\mu$ m sections were cut for either immunohistochemistry or *in situ* hybridization performed as described previously (4–7).

Antibodies used for immunohistochemistry were: (a) rabbit anti-mouse type IV collagen (BioDesign International, Kennebunk, ME), which stains basement membranes, including those of the microvasculature; and (b) an antibody to the NH<sub>2</sub>-terminal peptide (amino acids 1–25) of rat VPF/VEGF, kindly provided by Dr. Janice Nagy (Beth Israel Hospital, Boston, MA) (60, 61). Probes for *in situ* hybridization were synthesized by transcription of sense or antisense RNAs from either pGEM or pBluescript II plasmids containing the following inserts: mouse VPF/VEGF cDNA, 300 bp; SV40 small t intron and polyadenylation sequence, 600 bp; mouse flt-1 cDNA fragment, 545 bp; and mouse flk-1 fragment, 330 bp (mouse flt-1 and flk-1 clones were a kind gift from Clive Wood, Genetics Institute, Cambridge, MA). All of the above probes were specific and controlled for by the use of sense probes in the same experiment (4–7).

## RESULTS

**Screening for Human Tumor Cell Lines That Expressed Little or No VPF/VEGF mRNA Constitutively.** Recognizing that the great majority of tumorigenic cell lines overexpress VPF/VEGF constitutively (8, 13), we screened a number of established human tumor lines in search of cells that produced minimal amounts of VPF/VEGF mRNA. Northern analysis revealed that, in contrast to the majority of cell lines tested (U373, HT1080, HT29, T24, C6, NB41, and others), cultured M21 and SK-MEL-2 melanoma cells expressed very low levels of VPF/VEGF mRNA (Fig. 1). M21 cells expressed slightly more VPF/VEGF mRNA than did SK-MEL-2 cells and also expressed detectable levels of the VPF/VEGF receptor, KDR, whereas SK-MEL-2 cells did not. Expression of VPF/VEGF receptors is normally confined to vascular endothelium, although KDR expression has been reported previously on other human melanoma cell lines (67).

VPF/VEGF mRNA expression is up-regulated in many cell lines by hypoxia (29–37); this is a significant consideration because tumor cells growing *in vivo* are likely subjected to at least some degree of hypoxia. Therefore, to measure VPF/VEGF mRNA expression under conditions that more closely approximate those occurring *in vivo*, we

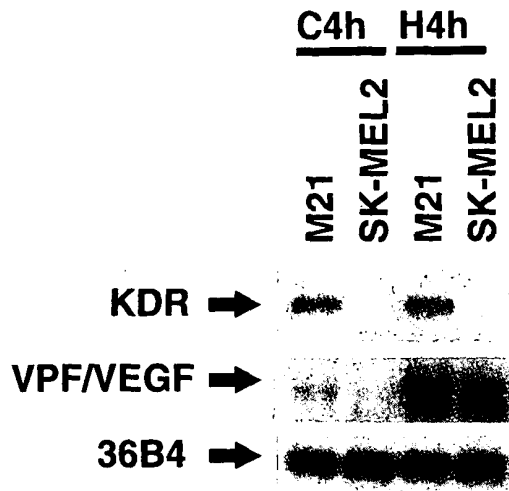


Fig. 1. Expression of endogenous VPF/VEGF mRNA (human, doublet at 4.2 and 3.7 kb) and KDR mRNA (7.5 kb) by two human melanoma cell lines, M21 and SK-MEL-2, was evaluated by Northern blotting. Cells were cultured for 4 h under normoxic (C) or hypoxic (H) conditions prior to RNA extraction. The 36B4 ribosome-associated protein mRNA (1.6 kb) was probed as a control for RNA loading, blotting, and hybridization.

cultured M21 and SK-MEL-2 cells under hypoxic conditions for periods of 4 and 24 h. Under these conditions, VPF/VEGF mRNA expression was strikingly and progressively up-regulated in M21 cells (7-fold at 4 h hypoxia as determined by phosphor imaging), whereas expression of the VPF/VEGF receptor KDR was oppositely regulated, falling below detectable levels after 24 h of culture (data not shown). VPF/VEGF mRNA expression was also up-regulated in SK-MEL-2 cells subjected to hypoxic culture conditions (4-fold at 4 h), but the amounts of VPF/VEGF mRNA induced were lower (by a factor of 3-fold at 4 h) than in comparably cultured M21 cells. Nonetheless, when SK-MEL-2 cells were subjected to prolonged hypoxic culture for 24 h, their expression of VPF/VEGF approached that of M21 cells (data not shown). In addition, KDR expression remained undetectable in SK-MEL-2 cells cultured under hypoxic conditions.

Measurements of secreted VPF/VEGF protein were consistent with results obtained by Northern analysis. Thus, supernatants from M21 cells cultured for 18 h under hypoxic conditions contained 67 pM VPF/VEGF as compared with only 13 pM in supernatants from similarly cultured SK-MEL-2. For reference, concentrations of VPF/VEGF necessary to regulate endothelial cell growth promotion and altered gene expression fall in a range between 22 and 200 pM (14, 16, 23).

These molecular findings were consistent with earlier studies from our laboratory with M21 and SK-MEL-2 cells. We had demonstrated previously that both cell lines grew at equivalent rates *in vitro* and formed tumors in immunodeficient nude mice; however, the tumors formed by M21 cells were well vascularized and exhibited minimal necrosis, whereas those formed by SK-MEL-2 cells were smaller, less well vascularized, and exhibited extensive necrosis (68). Therefore, based on these findings, and particularly on the lower baseline of VPF/VEGF mRNA expression, its lesser inducibility by hypoxia and the lack of KDR expression under both normoxic and hypoxic conditions, we selected the SK-MEL-2 cell line for transfection experiments.

**VPF/VEGF mRNA Expression in SK-MEL-2 Cells Transfected with Murine Sense or Antisense VPF/VEGF cDNA.** SK-MEL-2 cells were stably transfected with the cDNAs encoding either sense or antisense orientations of the mouse 164-amino acid VPF/VEGF

isoform under the control of a constitutive expression vector (see "Materials and Methods"). As a control, other SK-MEL-2 cells were transfected with vector alone without a VPF/VEGF insert. Pools of the resulting transfectants were then cultured under normoxic conditions, and total RNAs were extracted for Northern analysis (Fig. 2).

As in the parental SK-MEL-2 population, transfectants expressed endogenous human VPF/VEGF mRNA of 4.2 and 3.7 kb, whereas the vector-directed murine VPF/VEGF mRNA (both sense and antisense) formed a readily distinguishable band at 1.9 kb. SK-MEL-2 cells transfected with vector alone (SK-MEL-2 N1 cells) expressed only small amounts of endogenous human VPF/VEGF mRNA and, as expected, no detectable murine VPF/VEGF mRNA. However, two pooled stably transfected cell lines containing the murine VPF/VEGF sense (SK-MEL-2 V+1 or V+2) or antisense (SK-MEL-2 V-1 or V-2) insert expressed high levels of vector-derived mRNAs at 1.9 kb detected by double-stranded murine VPF/VEGF cDNA probe. Of interest, levels of endogenous human VPF/VEGF mRNA expression were also increased somewhat in several sense- and antisense-expressing transfectants [as much as a 3-fold induction above vector-only transfected control cell (N1) levels, as determined by phosphorimager analysis].

**Immunoreactive and Bioactive VPF/VEGF Protein Secreted by Transfected Cells.** To determine whether the various transfectants also expressed VPF/VEGF at the protein level, cells were cultured for 24 h under normoxic conditions in serum-free medium. Serum- and cell-free supernatants were then collected and subjected to immunoassays that separately measured VPF/VEGF of mouse and human origin (Table 1). High levels of murine, but not human, VPF/VEGF

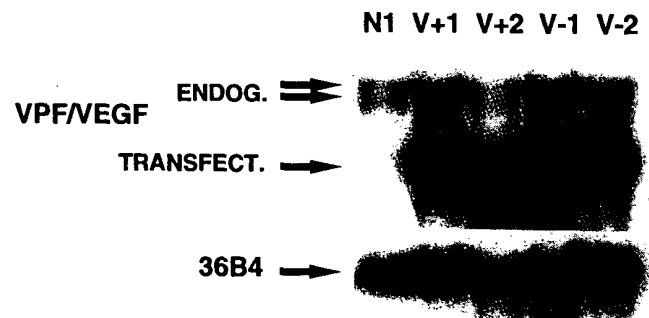


Fig. 2. Northern blot of SK-MEL-2 cell pools stably transfected to express sense (V+1 and V+2) or antisense (V-1 and V-2) mouse VPF/VEGF mRNAs. N1, SK-MEL-2 cells were transfected with CMV vector alone. As in Fig. 1, endogenous (ENDO.) human VPF/VEGF mRNA transcripts were detected as a doublet at 4.2 and 3.7 kb, and vector-driven murine VPF/VEGF transcripts were detected as a single 1.9-kb band (TRANSFECT.). Control hybridization was performed with the 36B4 probe.

Table 1 Correlation of VPF/VEGF secretion *in vitro* and tumor growth in the s.c. or subdermal space of SCID mice

Transfected SK-MEL-2 tumor cells	VPF-VEGF protein determined by immunoassay (pM)		S.C. tumor weight (mg) m $\pm$ SEM (n = 8)	Subdermal tumor weight (mg) m $\pm$ SEM (n = 6)
	Mouse	Human		
N1	<sup>a</sup>	<sup>a</sup>	38 $\pm$ 6.6	17 $\pm$ 2.7
V + 1 <sup>b</sup>	802,670 <sup>c</sup>	<sup>a</sup>	111 $\pm$ 33 <sup>d</sup>	216 $\pm$ 60 <sup>c</sup>
V-1	<sup>a</sup>	<sup>a</sup>	18 $\pm$ 1.9	4 $\pm$ 0.97

<sup>a</sup> Below assay detection limit.

<sup>b</sup> In a single experiment, immunoreactive VPF/VEGF was measured to be 727 pM in V + 2 cell culture supernatants, similar to that of V + 1 culture supernatants.

<sup>c</sup> Data from two separate experiments.

<sup>d</sup> Significantly different from N1 and V-1 at *P* < 0.01 by Tukey-Kramer test.

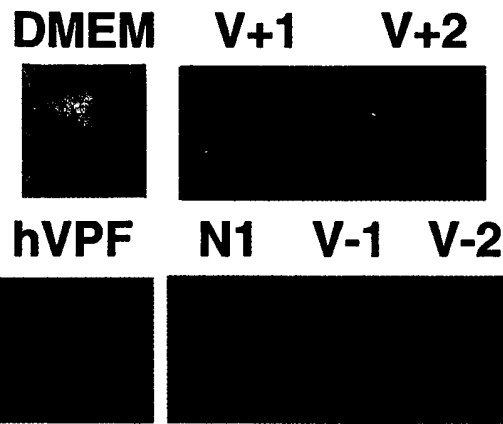


Fig. 3. Biological activity of culture supernatants prepared from transfected SK-MEL-2 cells as determined in the Miles assay. Mice injected i.v. with Evans blue dye were injected intradermally with serum- and cell-free conditioned medium (24 h) prepared from N1, V+1, V+2, V-1, and V-2 cell pools, and bluing was assessed 25 min later. Recombinant human VPF/VEGF (hVPF; 0.02  $\mu$ M) was injected as a positive control. Supernatants from V+1 and V+2 cells gave strong positive results, whereas those from N1, V-1, and V-2 cells were at very low or background levels.

were detected in SK-MEL-2 V+1 and V+2 cells transfected with sense VPF/VEGF cDNA. However, assays of culture supernatants prepared from SK-MEL-2 N1, V-1, or V-2 cells revealed little or no detectable human or mouse VPF/VEGF.

Culture supernatants were also tested in the Miles assay for biological activity in promoting extravasation of circulating macromolecules. Consistent with the immunoassay data, supernatants from cultured SK-MEL-2 V+1 and V+2 cells that were injected into normal mouse skin generated striking effusions of Evan's blue dye (Fig. 3). In contrast, supernatants from SK-MEL-2 N1 cells generated only a minimal positive response, and those from SK-MEL-2 V-1 and V-2 cells elicited insignificant dye extravasation that was indistinguishable from control sites injected with DMEM.

**Growth and Morphology of Transfected SK-MEL-2 Cells Growing as Xenografts in SCID Mice.** The three types of transfected cells were tested for their capacity to grow in the s.c. or subdermal space of immunodeficient nude or SCID mice, respectively. SK-MEL-2 V+1 cells grew much faster and formed larger tumors than either SK-MEL-2 N1 or SK-MEL-2 V-1 cells (Table 1).

In addition to differences in size, the several transfectants were found to grow in different histological patterns (Fig. 4). Like the parental cell line, SK-MEL-2 N cells formed tumors with moderate amounts of central necrosis (necrosis representing 30–50% of tumor volume in four separate tumors). In contrast, SK-MEL-2 V+ cells, which overexpressed immunoreactive and bioactive mouse VPF/VEGF, formed larger tumors with little or no necrosis (<5% in four separate V+1 tumors and <10% in each of four V+2 tumors). Finally, SK-MEL-2 V- cells, which overexpressed antisense VPF/VEGF, formed smaller tumors with extensive areas of central necrosis, surrounded by only a thin rim of viable tumor cells. In each of four V-1 tumors, ~80% of the tumor mass was necrotic; in each of four V-2 tumors, necrosis accounted for ~60% of tumor mass.

**Expression of VPF/VEGF and Its Receptors by Transfected SK-MEL-2 Cells Growing as Xenografts in SCID Mice.** We performed *in situ* hybridization to determine whether the various transfected cell populations maintained the patterns of murine VPF/VEGF sense or antisense mRNA expression *in vivo* that had been observed in culture. As shown in Fig. 5, neoplastic V+1 cells expressed high levels of murine VPF/VEGF mRNA. Using the same antisense probe, V-1 cells did not express murine VPF/VEGF mRNA above background levels; however, high levels of antisense VPF/VEGF mRNA expression were detected when these cells were hybridized with a VPF/VEGF sense riboprobe.

We also evaluated the expression of the two recognized VPF/VEGF receptors, *flt-1* and the KDR mouse homologue *flk-1*, by *in*

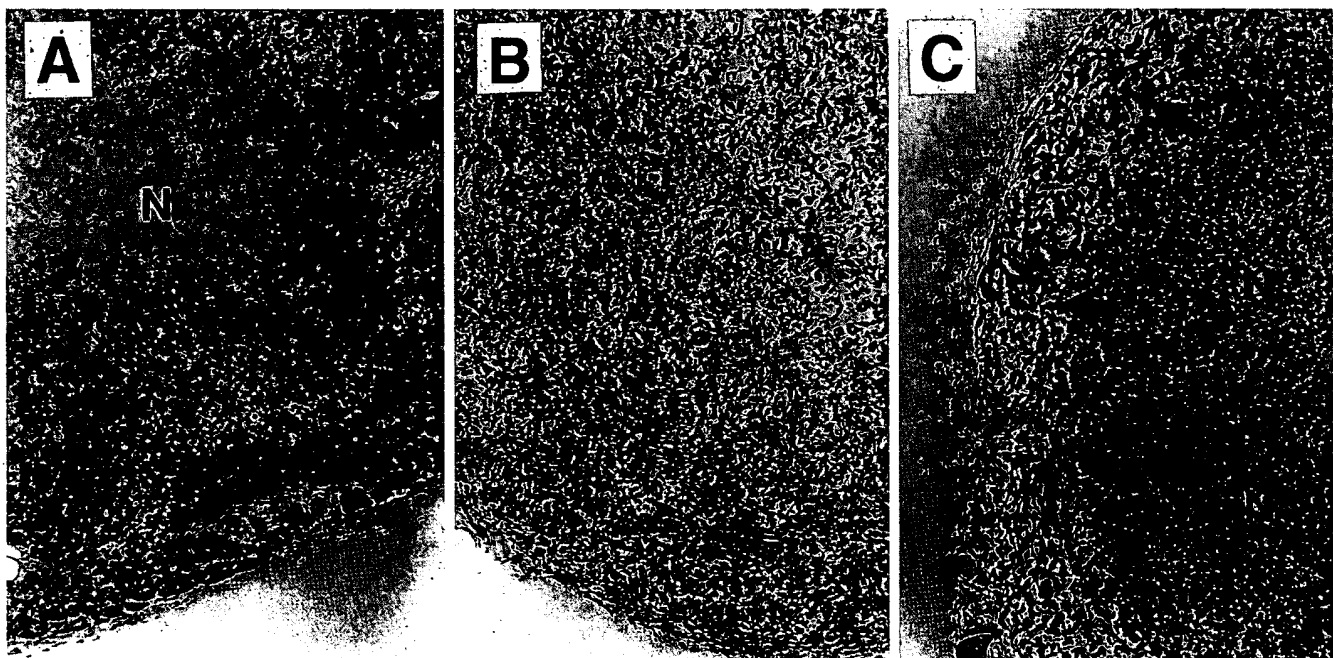
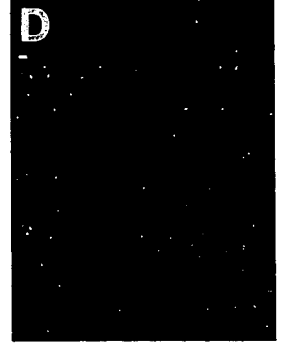
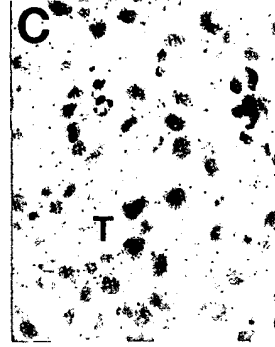
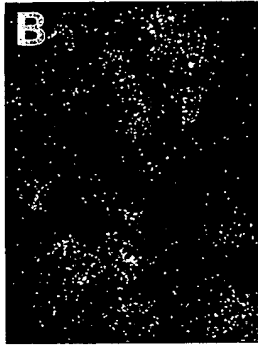


Fig. 4. Histology of tumors that developed 14 days after s.c. injection of SK-MEL-2 transfected cells into nude mice. A, N1 cells (vector-only transfectants) yielded small, poorly vascularized tumors with necrotic (N) centers. B, V+1 cells (mouse sense VPF/VEGF transfectants) yielded larger, well-vascularized tumors with minimal necrosis. C, V-1 cells (mouse antisense VPF/VEGF transfectants) developed small, poorly vascularized tumors comprised of a thin shell of viable tumor cells surrounding a large central necrotic core. Arrows, demarcation of border between viable (peripheral) tumor and central necrosis.  $\times 115$ .

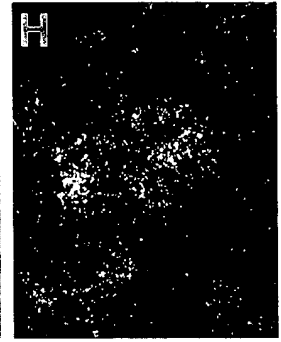
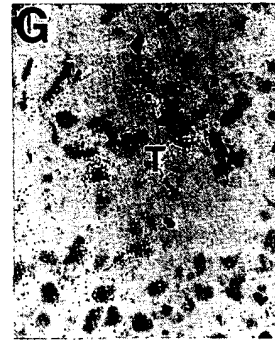
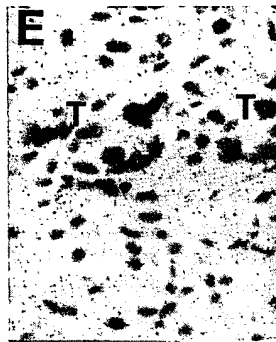
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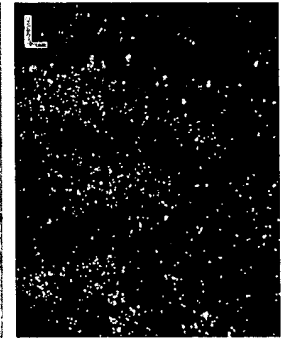
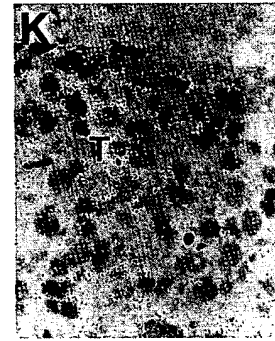
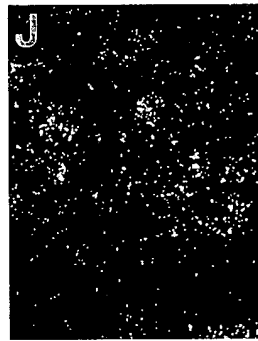
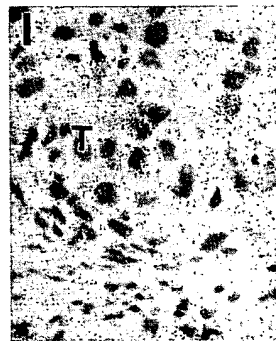
VPF AS



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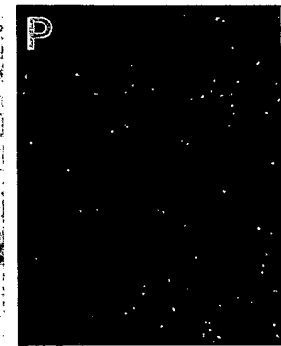
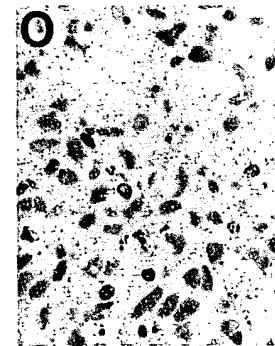
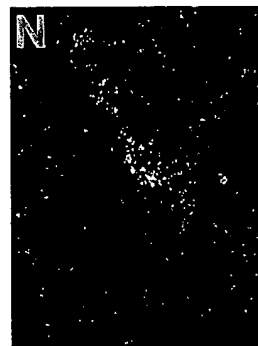


Fig. 5. *In situ* hybridization of SK-MEL-2 V+1 and SK-MEL-2 V-1 tumors (T) growing in the s.c. space of nude mice and photographed, alternatively, in bright field or with dark field. Sections were hybridized with RNA probes as follows: antisense (AS) VPF/VEGF (A-D); sense (S) VPF/VEGF (E-H); antisense SV40 t-intron/PA (I-L); and antisense flk-1 (M-P). SK-MEL-2 V+1 tumor cells consistently overexpressed the following mRNAs: VPF/VEGF (A and B), SV40 t-intron/PA (I and J), and flk-1 (M and N). In contrast, SK-MEL-2 V-1 overexpressed SV40 t-intron/PA mRNA (K and L) and antisense VPF/VEGF mRNA (G and H) but did not express detectable levels of sense VPF/VEGF (C and D) or of flk-1 mRNA (O and P). Arrow in M, tumor blood vessel.

*situ* hybridization (Fig. 5). As has been found to be typical of most human and animal tumors that have been studied to date, mRNAs encoding the VPF/VEGF receptor KDR/flk-1 were highly overexpressed in tumors formed by V+1 cells; KDR/flk-1 mRNA was confined to endothelial cells lining tumor microvessels, and none

was found associated with tumor cells. In contrast to V+1 tumors, the relatively avascular tumors formed by SK-MEL-2 V-1 cells expressed only low levels of KDR/flk-1 mRNA, confined to microvessel endothelial cells. This was also the case for control transfected SK-MEL-2 N1 tumors (data not shown). The second

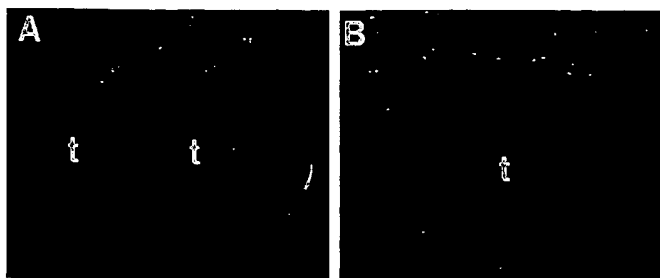


Fig. 6. Comparison of microvascular permeability in tumors formed by VPF/VEGF-hypersecreting SK-MEL-2 V+1 cells and vector-only transfected SK-MEL-2 N1 cells. Five days after injection of  $1 \times 10^6$  tumor cells into the s.c. space of SCID mice, 3 mg FITC-D (average  $M_r$  145,000) was injected i.v.; 30 min later, tumors were harvested, fixed, and processed for paraffin sections under anhydrous conditions. Fluorescence microscopy revealed a bright rim of FITC-D extravasation around SK-MEL-2 V+1 tumors (A) and very little fluorescence around N1 tumors (B). *t*, tumor nodule. A,  $\times 250$ ; B,  $\times 430$ .

VPF/VEGF receptor, *flt-1*, was expressed in vascular endothelium of V+1 tumors but only at background levels in tumors formed by V-1 cells.

**Microvascular Hyperpermeability and Angiogenesis Induced by VPF/VEGF Transfected Cells Growing in SCID Mice.** The three types of transfected cells were injected s.c. into SCID mice. At 24 and again at 72 h, Coomassie blue dye was injected i.v., and bluing was assessed at tumor cell injection sites. Prominent bluing was observed at sites of V+ cell injection at both time intervals, whereas only minimal bluing was observed at sites of N1 cell injection (data not shown). Sites injected with SK-MEL-2 cells transfected with the antisense VPF/VEGF vector (V- cells) showed only a background level extravasation of Coomassie blue at all times up to 96 h after s.c. tumor cell injection.

To determine more precisely the nature and extent of vascular hyperpermeability induced by the various transfected SK-MEL-2 tumor cells, immunodeficient mice bearing 5-day tumors were injected i.v. with  $M_r$  145,000 FITC-D, and the tumors were harvested for histological study 1 h later. As shown in Fig. 6, the microvessels surrounding the growing V+ tumors exhibited extensive microvascular hyperpermeability to FITC-D, whereas much less leakage was observed in tumors formed by N cells. Antisense-expressing V- cells had not formed palpable tumor nodules by day 5 and did not give evidence of vascular hyperpermeability (data not shown).

To evaluate the level of tumor angiogenesis induced by the various SK-MEL-2 transfectants, sections of the three types of tumor xenograft were immunostained with anti-type IV collagen antibodies, which highlighted microvessels by labeling their basement membranes. As shown in Fig. 7, tumors formed by V+1 cells were highly vascular, those formed by V-1 cells were poorly vascularized, and those formed by N1 cell transfectants exhibited intermediate vascularity, similar to that of parental (untransfected) SK-MEL-2 tumor cells. Immunohistochemistry was also used to assess mouse VPF/VEGF protein in V+1 tumors. Abundant mouse VPF/VEGF was detected in the sense VPF/VEGF-expressing tumors; as described previously (4, 5, 7, 9, 60, 61), staining for VPF/VEGF was observed both in tumor cells and in adjacent microvessels. In contrast, control N1 tumors did not stain with antibodies to mouse VPF/VEGF (Fig. 7).

**Ability of Transfected SK-MEL-2 Cells to Form Lung Colonies following i.v. Injection.** In a first experiment,  $1 \times 10^5$  N1, V+1, V+2, V-1, or V-2 cells were injected i.v. into groups of four SCID mice each; three weeks later, lungs were harvested, and random histological sections were prepared and studied by low power microscopy. A total (mean  $\pm$  SEM) of  $18.5 \pm 8.4$  and  $14.5 \pm 3.4$  tumor

nodules were found per low power field (final magnification,  $\times 40$ ) in V+1 and V+2 injected mice, respectively. In contrast, only two individual tumor nodules were found in similar lung sections taken from the 12 mice receiving N1 (one), V-1 (none), and V-2 (one) tumor cells.

In a second experiment, groups of SCID mice (four each) were injected i.v. with  $2 \times 10^5$  V+1, V-1, or N1 cell pools. Three weeks later, lungs were harvested, and surface tumor nodules were counted. SK-MEL-2 V+1 cells formed many more lung colonies than did N1 cells:  $216 \pm 29.9$  versus  $4.0 \pm 1.2$ . In addition, the individual colonies formed by V+1 cells were substantially larger and better vascularized than those formed by N cells (Fig. 8). In contrast, i.v.-injected V-1 cells did not form any lung colonies at the macroscopic level.

## DISCUSSION

The data presented here provide strong direct evidence that VPF/VEGF has an important role in facilitating tumor growth *in vivo* by stimulating tumor angiogenesis. SK-MEL-2 melanoma cells were selected for study because, of all tumorigenic cell lines tested, they expressed the least amount of VPF/VEGF when growing as solid tumors *in vivo* or when cultured under normoxic or hypoxic conditions. Parental SK-MEL-2 melanoma cells formed small, slowly growing s.c. or subdermal tumors in immunodeficient mice that were characterized by necrosis and low vascular density. When stably transfected with mouse sense VPF/VEGF cDNA, however, these V+ cells formed tumors that grew much more rapidly, developed an adequate blood supply, and exhibited little ischemic necrosis. These results stand in contrast to the behavior of SK-MEL-2 cells transfected with vector alone (N1 cells) or transfected with antisense VPF/VEGF cDNA (V- cells). N1 cells behaved much like the parental line *in vivo*, whereas V- cells, which expressed still lower levels of bioactive human VPF/VEGF as determined in the Miles assay, formed very small, poorly vascularized tumors with extensive necrosis. Finally, following i.v. injection, V+ cells formed numerous lung tumor nodules, more than 50 times more than were formed by N1 cells. Comparable numbers of V- cells formed very few or no detectable lung nodules following i.v. injection. Taken together, these data emphasize the importance of VPF/VEGF in primary tumor growth and point to a role for VPF/VEGF in the later phases of the metastatic process.

Our results complement those reported recently by Potgens *et al.* (69). These workers also found that hypoxia stimulated VPF/VEGF expression in several human melanoma cell lines. Moreover, tumors having low metastatic potential expressed low levels of VPF/VEGF in culture, whereas melanoma lines that formed tumors with high metastatic potential expressed high levels of VPF/VEGF in culture. However, these differences in VPF/VEGF expression disappeared when tumors were grown *in vivo*. The authors attributed their results to up-regulation of VPF/VEGF expression *in vivo* by hypoxia. They also demonstrated a change in tumor vascular architecture when a melanoma cell line with hypoxia-inducible VPF/VEGF expression was engineered into a line with constitutive VPF/VEGF expression.

Several features of our experiments deserve further comment. A first point of interest is the difficulty we encountered in finding a tumorigenic cell line that did not express abundant VPF/VEGF constitutively. Screening of some 15 human and rodent lines revealed that all except SK-MEL-2 melanoma cells expressed substantial levels of VPF/VEGF mRNA when cultured under standard normoxic conditions and that expression was further up-regulated, although to varying degrees, when any of these cells were cultured under hypoxic conditions of the type likely to occur when tumors grow *in vivo*. In

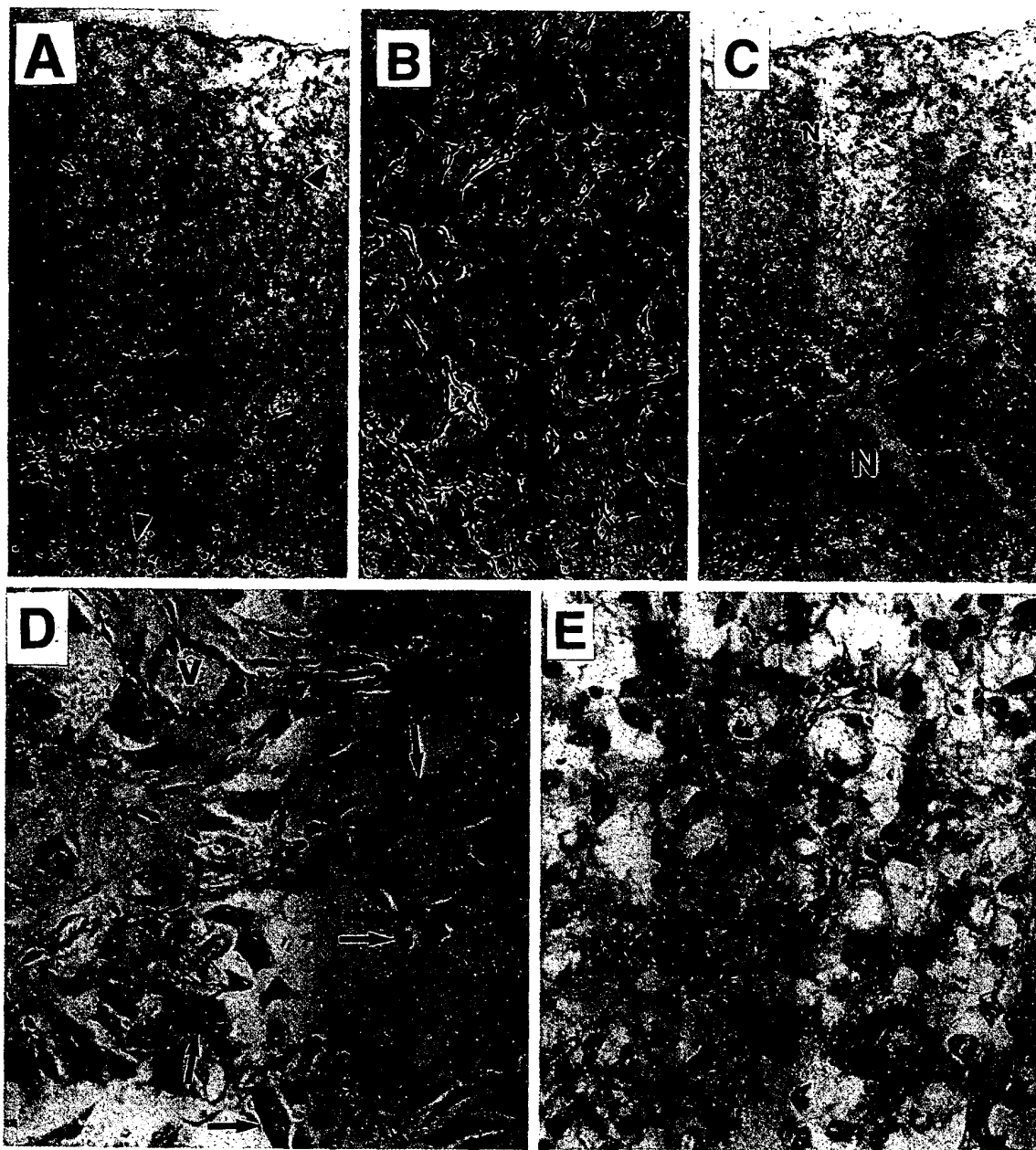


Fig. 7. Collagen type IV (A-C) and VPF/VEGF immunostaining (D and E) of tumors formed by SK-MEL-2 cells stably transfected with vector only (N1), with sense mouse VPF/VEGF (V+1) or with antisense mouse VPF/VEGF (V-1). Collagen type IV immunostaining was used to identify tumor microvessels by staining their basement membranes. A, N1 tumors had relatively few vessels and were characterized by small foci of necrosis (arrowheads). B, V+1 tumors were highly vascular and exhibited little or no necrosis. C, V-1 tumors exhibited fewer blood vessels than N1 tumors and more extensive central necrosis (N); even the peripheral rim of viable tumor (upper) contains focal necrosis (N). D, immunostaining of SK-MEL-2 V+1 tumor with an antibody to the NH<sub>2</sub>-terminal peptide of murine VPF/VEGF reveals cytoplasmic staining of tumor cells (arrows) as well as the endothelium of blood microvessels (v). E, in contrast, an SK-MEL-2 N1 tumor exhibits no significant staining for mouse VPF/VEGF. A-C,  $\times 270$ ; D and E,  $\times 670$ .

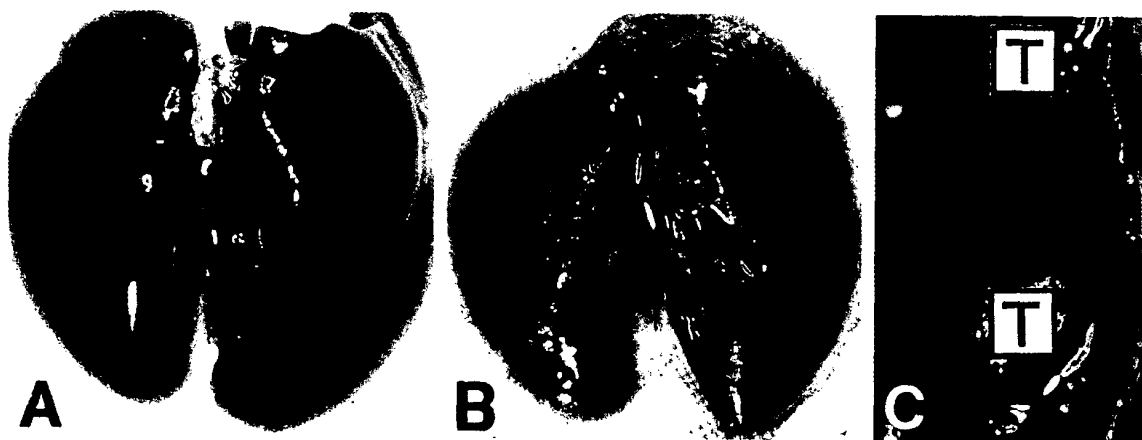


Fig. 8. Lung tumor colonies following i.v. injection of  $2 \times 10^5$  SK-MEL-2 N1 (A) or V+1 (B and C) cells. SK-MEL-2 V+1 cells produced numerous macroscopic lung colonies (B and C) labeled as T, whereas SK-MEL-2 N1 cells produced many fewer such colonies and in this particular lung, none.

fact, we were unable to identify a tumorigenic cell line that did not express detectable levels of VPF/VEGF mRNA when cultured under hypoxic conditions and therefore, to conduct this study, we were obliged to use the SK-MEL-2 melanoma cell line that expressed relatively smaller (but still sizable) amounts of VPF/VEGF mRNA as compared with the other cell lines we tested. These findings are in agreement with studies of autochthonous and metastatic human tumors, the great majority of which express abundant VPF/VEGF mRNA *in situ* (8, 13).

A second point to be emphasized is that in comparing VPF/VEGF expression among different cell lines, it is important to test cells under both normoxic and hypoxic culture conditions. It is now well known that VPF/VEGF expression is strikingly up-regulated by hypoxia in nearly all cultured cells, including primary cultures of many normal cells (even including endothelial cells<sup>4</sup>). To the extent that tumors growing *in vivo* are hypoxic, cell cultures at reduced oxygen tension may give a more realistic picture of tumor cell VPF/VEGF expression than that obtained when cells are cultured under the usual normoxic conditions. Furthermore, our findings raise the possibility that VPF/VEGF expression may be requisite for tumorigenicity; as noted, we have yet to identify a tumorigenic cell line that does not express detectable levels of VPF/VEGF under hypoxic conditions. We have, however, described two relatively uncommon autochthonous human tumors (lobular carcinoma of the breast and papillary renal carcinomas) that do not strongly express VPF/VEGF mRNA as determined by *in situ* hybridization (5, 6). Of note, both of these tumors are relatively avascular as compared with the more common carcinomas that arise in breast and kidney and that strongly overexpress VPF/VEGF.

A third point of note is that expression of the mRNAs encoding the VPF/VEGF receptors flt-1 and KDR/flk-1 paralleled that of VPF/VEGF mRNA expression and overall vascular density. Thus, flt-1 and KDR/flk-1 mRNAs were strongly expressed by vascular endothelium in the numerous new vessels elicited by VPF/VEGF overexpressing V+ tumor cells, whereas neither was abundantly expressed in tumors formed from V- cells. The mechanisms regulating the expression of VPF/VEGF receptor mRNAs are not well understood. Hypoxic culture conditions (24 h) increased the expression of flt-1 mRNA by dermal microvascular endothelial cells, whereas expression of KDR was reduced to undetectable levels,<sup>5</sup> similar to the regulation we observed in the M21 tumor cells. Thus, in melanoma cells as in cultured endothelial cells, hypoxia represses KDR/flk-1 expression.

A fourth point is that transfection of SK-MEL-2 cells with the mouse antisense VPF/VEGF cDNA led to reduced expression of endogenous human VPF/VEGF *in vitro*. Moreover, these V- cells induced significantly less angiogenesis than either the parental strain or than N1 cells transfected with vector alone, and presumably as a consequence, formed small, slowly growing tumors with extensive necrosis when implanted in immunodeficient mice. These results provide evidence that the human VPF/VEGF secreted by parental SK-MEL-2 cells and by N1 vector transfectants is effective at inducing angiogenesis in mice and that overexpression of a mouse antisense VPF/VEGF construct can inhibit expression of endogenous human VPF/VEGF.

Two additional points deserve mention. The first is the close correlation that we found between VPF/VEGF expression, microvascular hyperpermeability, and tumor-induced angiogenesis. Thus, V+ cells formed tumors that induced striking microvascular hyperpermeability and angiogenesis, whereas N1 and V- transfectants formed smaller, less vascular tumors with much less microvascular hyperpermeability and angiogenesis. We have sum-

marized evidence elsewhere that microvascular hyperpermeability represents an early and perhaps a requisite stage in the sequence of events leading to angiogenesis (8), and the data presented here are consistent with this hypothesis. Although numerous angiogenesis factors have been described, only VPF/VEGF is known to be capable of inducing microvascular hyperpermeability by a direct action on vascular endothelium. Second, our data indicate that VPF/VEGF plays a critical role in the formation of lung tumor colonies when tumor cells are injected i.v. The i.v. injection of small numbers ( $1-2 \times 10^5$ ) of V+ tumor cells led to the formation of large numbers of lung metastases in SCID mice, some 50-fold more numerous than developed following the injection of the same number of N1 cells. Furthermore, i.v. injection of similar numbers of V- cells failed to induce significant numbers of lung metastases. We considered the possibility that these results might simply indicate that tumor cells expressing VPF/VEGF possess a growth advantage *in vivo* so that they formed larger and therefore more easily recognizable tumors than did cells transfected with vector alone or with antisense VPF/VEGF cDNA. In fact, individual lung tumors formed by V+ cells were considerably larger than those formed by N1 cells, whereas V- tumor cells only formed only rare, microscopically detectable tumors. One likely reason for this finding is that the lung is an exceptionally well-oxygenated tissue, and in this environment, hypoxia-mediated induction of VPF/VEGF production by SK-MEL-2 cells may be less likely to occur than in the s.c. or subdermal spaces; in this environment, constitutive overexpression of VPF/VEGF may be required to form lung colonies.

Presently uncertain is the mechanism(s) by which VPF/VEGF overexpression favors the formation of tumor colonies in the lung. The lung colonization assay collectively measures several of the important steps in tumor metastasis including tumor cell survival in the circulation, extravasation from microvessels, and the capacity for cell growth in a distant site. Because high levels of VPF/VEGF expression favor SK-MEL-2 tumor cell growth in both s.c. and subdermal sites, we expect that this property is also important in lung colonization. Koop *et al.* (70) have reported recently that the majority of mouse B16F10 melanoma cells injected i.v. survive in the circulation and extravasate; therefore, at least in their system, the critical factor in determining the efficiency of lung colonization was the ability of cells to grow at stages of the metastatic process that followed after extravasation. Whether VPF/VEGF overexpression also favors cell survival in the circulation or tumor cell extravasation in addition to postextravasation tumor growth awaits further experimentation.

## ACKNOWLEDGMENTS

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<sup>4</sup> M. Detmar, unpublished data.

<sup>5</sup> M. Detmar, unpublished results.

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